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REVIEW ARTICLE

# Hepatitis A infections from food

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## Summary

Food contaminated by hepatitis A virus (HAV) is responsible of the 2–7% of all HAV outbreaks worldwide. This review provides a description of the HAV characteristics, its infectivity and epidemiological features. In addition, this review compiles existing original papers reporting HAV prevalence, viral titres in foodstuffs and the risk associated with food contamination. The purpose of this revision is to conduct a structured and systematic review of the published molecular procedures for HAV detection in food, including the assessment of its infectivity.

## Introduction

### Classification and structure

Hepatitis A virus (HAV) belongs to the Picornaviridae family and it is classified under the *Hepatovirus* genus. Unlike hepatitis B and C viruses, which are sexual and blood transmitted and responsible for 96% of all hepatitis mortality, HAV does not cause chronic liver disease and it is mainly transmitted through the faecal–oral route. However, HAV can cause acute liver failure, which may be fatal. WHO estimated that in 2016, 7134 people died from HAV worldwide (accounting for 0·5% of the mortality due to viral hepatitis; WHO 2017).

HAV is a small virus, 27–32 nm diameter, with a linear, positive-sense, single-stranded RNA genome of about 7·5 kb in length. There are two forms of infectious virus: naked, nonenveloped HAV virions shed in the stools and quasi-enveloped virions (eHAV) that are secreted nonlytically from infected cells. eHAV are found in the blood of infected patients, or in the supernatant of infected cell cultures (Lemon *et al.* 2017).

The HAV genome is organized in two noncoding regions at each of the ends, 5′ and 3′. The P1 region encodes the structural proteins VP1, VP2, VP3 and a putative VP4, and the P2 and P3 regions encode nonstructural proteins associated with replication. The antigenic structure of the capsid is highly conserved, and there is only a single serotype of human HAV. However, based on the analysis of a 168-nucleotide segment of the VP1-2A region, HAV is classified in six genotypes. Genotypes I, II and III, have human origin and are divided into subtypes A and B, while genotypes IV, V and VI cause infections in simians (Desbois *et al.* 2010).

### Features of HAV infection

The course of hepatitis A ranges from mild to severe, and is typically more severe in adults than in children (<6 years), that are often asymptomatic. Common symptoms associated with acute HAV infection are loss of appetite, fever, headache, nausea, diarrhoea, abdominal discomfort, anorexia, myalgia, dark-coloured urine and jaundice. HAV infection develops a lifelong immunity and does not result in chronic infection or chronic liver disease (Lemon *et al.* 2017).

Adults with hepatitis A illness usually develop symptoms after a long incubation period of 14-28 days (up to 50 days) after the exposure, and a high numbers of virus particles are excreted reaching the maximum of up to $10^{11}$ genome copies (gc) per gram of faeces just before the onset of symptoms. The fatality rate in HAV infections is lower than 0·1%, although in elderly it may rise up to 1·8–5·4% (1·8–5·4%) (Bosch *et al.* 2016).

### Routes of transmission and epidemiology

HAV is excreted in faeces and the main route of transmission is from person to person by the faecal–oral route

mainly among close contacts, including household and sexual contacts of people infected with HAV. Over the past three decades men who have sex with men (MSM) are particularly at risk for HAV infection and several outbreaks among MSM have been reported across Europe, North-America and Australia (Ndumbi *et al.* 2018). Also, HAV can occasionally be acquired through blood transfusions (Noble *et al.* 1984). In addition, HAV can be transmitted by ingestion of contaminated food or water and closed environments, restaurants, and caterings results the most common settings of HAV outbreaks (FDA 2020).

Hepatitis A epidemiology differs considerably among regions worldwide and it is changing according to the economic development, as much is the improvement of hygienic and sanitary conditions as lower is the occurrence of outbreaks. Additionally, the number of reported cases has declined substantially in countries with effective programs of immunization with a HAV vaccine. As example, in US HAV incidence rates decreased more than 95% since 1995, when vaccine first became available, to 2011 and fluctuations are due to occasional outbreaks linked to imported food and among nonimmune MSM. In 2017, more than 3366 reported cases and almost 6700 estimated cases were linked to a large person-to-person outbreak occurred among drug-addicted people and individuals experiencing homelessness (CDC 2017). In highly endemic areas, the majority of individuals are infected in early childhood and virtually all adults are immune. However, HAV infections in industrialized countries have become less common as a result of increased living standards. In these low–moderate endemic countries, only few people are infected in early childhood, and the majority of adults remain susceptible to infection lifelong. In such scenarios, outbreaks of hepatitis A are often reported and recognized as a serious public health issue.

## HAV in food

Contamination of food with HAV can occur at any point of the food chain from farm to fork. The contact with incorrectly treated sewage or sewage-polluted water, with infected food handlers and, to a lesser extent, with contaminated surfaces represent the most common routes of HAV contamination in food. Approximately 2–7% of all HAV outbreaks worldwide can be attributed to contaminated food. As reported earlier (Sánchez 2015), closed setting environments, restaurants and catering services are most common places of HAV outbreaks.

Ready-to-eat foods that do not undergo further processing, bivalve molluscs, fresh leafy greens, fresh and frozen berries have frequently been implicated in HAV outbreaks, often from imported foods (Table 1). Bivalve molluscs are filter feeders that ingest and pass out large quantities of water in order to screen out and consume tiny food particles. Most shellfish-borne outbreaks caused by HAV have been associated with the consumption of raw or under-cooked shellfish (Pintó *et al.* 2009; Pérez-Sautu *et al.* 2011; Boxman *et al.* 2016), usually harvested from waters affected by the discharge of treated and untreated sewage.

Several factors contribute to the risk of viral contamination of produce (vegetables and berries) during primary production, postharvest processing and distribution. Generally, agricultural products grow close to the ground, which increases their contacts with sewage-polluted irrigation water or contaminated biosolids. Furthermore, from harvest to consumption, produce undergo multiple manipulation processes, which increase the risk of their cross-contamination by infected handlers or contaminated surfaces.

In the case of HAV, the surveillance and reporting systems for systemic infections have been used to epidemiologically investigate and apply appropriate control measures.

Giving the global spread of HAV contaminated produce, it is not surprising that outbreaks occurred and some epidemiological investigations succeeded in tracing back the source of contamination. For instance, between 2013 and 2014, Europe experienced a large and prolonged food-borne multistate HAV outbreak associated with consumption of frozen berries accounting to more than 1589 cases and two deaths (Severi *et al.* 2015; Scavia *et al.* 2017). An overview of HAV outbreaks associated with different food is reported in Table 1.

The occurrence of food-borne HAV outbreaks is undeniably linked to the persistence and stability of infectious virus particles in water, soil, fomites and contaminated hands.

Like other human enteric virus, HAV has been demonstrated to retain its infectivity under different physical, chemical and biological conditions, resulting resistant to inactivation at high temperatures and on environmental fomites (reviewed by Sánchez and Bosch 2016). Thus, the effect of storage conditions and processing technologies to control HAV has been investigated in high-risk food matrices on a food safety perspective.

Refrigeration from 3 to 10°C does not guarantee HAV inactivation. HAV mantained its infectivity after 1 year of storage in bottled water stored at 4°C (Biziagos *et al.* 1988). Results on produce indicated that HAV survived on lettuce and carrots up to 4 days, on fennel up to 7 days (Croci *et al.* 2002), more than 20 days on green onions (Sun *et al.* 2012), and up to 42 days in packaged spinachs (Shieh *et al.* 2009). Similarly, freezing prevents HAV inactivation for up to 3 months on strawberries,

**Table 1** Foods associated with hepatitis A virus outbreaks in the last decade (2009–2019)

| Implicated food | Outbreak location | Year | Origin raw material | Number of cases | References |
|---|---|---|---|---|---|
| Pomegranate arils (frozen) | Canada | 2012 | Egypt | 9 | Swinkels *et al*. (2014) |
| | USA | 2013 | Turkey | 165 | Collier *et al*. (2014) |
| | Australia | 2018 | Egypt | 30 | Franklin *et al*. (2019) |
| Fresh dates | Sweden | 2018 | Iran | 27 | Müller *et al*. (2018) |
| Sun dried tomatoes | Australia, The Netherlands, France | 2009–2010 | | 308 | Petrignani *et al*. (2010), Gallot *et al*. (2011) |
| Frozen mix berries | Several European countries | 2013–2014 | Bulgaria, Poland | 1589 | Severi *et al*. (2015) |
| | USA | 2013 | Turkey | 162 | Collier *et al*. (2014) |
| Frozen strawberries | Several European countries | 2012–2013 | | | European Centre for Disease Prevention and Control and European Food Safety Authority (2013) |
| | Sweden, Austria | 2018 | Poland | 31 | Enkirch *et al*. (2018) |
| Bakery products | Germany | 2012 | Germany | 83 | Harries *et al*. (2014) |
| Scallops | Hawaii | 2016 | Philippines | 292 | Viray *et al*. (2018) |

blueberries, raspberries, parsley and basil (Butot *et al*. 2008).

In shellfish, HAV showed minimal infectivity reduction in refrigerated marinated mussels after 4 weeks (Hewitt and Greening 2004) and HAV sequences have been detected in oysters and clams implicated in outbreaks after long-term freezing (Sánchez *et al*. 2002; Shieh *et al*. 2007; Pintó *et al*. 2009).

Furthermore, HAV persists under low humidity conditions on lettuce, bell peppers, cantaloupe (Stine *et al*. 2005) and also on dried tomatoes (Petrignani *et al*. 2010; Gallot *et al*. 2011).

The effect of modified atmospheres (MAP) on HAV stability has been minimally investigated and a study on lettuce packaged under different carbon dioxide:nitrogen concentrations indicated that commercial MAP conditions do not affect HAV (Bidawid *et al*. 2001).

Other strategies to control HAV rely on food-grade compounds exerting antiviral activity that can be added directly to food and beverages or used to develop active packaging materials or edible coatings (D'Souza 2014; Randazzo *et al*. 2018a). Essential oils obtained from lemon, grapefruit, rosemary cineole, oregano and zataria as well as thymol and carvacrol, plant-derived monoterpenoid phenols, demonstrated antiviral activity and, interestingly, the formers used to control HAV in soft fruits (Sánchez and Aznar 2015; Sánchez *et al*. 2015; Battistini *et al*. 2019).

Similarly, grape seed extract and green tea extract reduced HAV infectivity (Su and D'Souza 2011; Randazzo *et al*. 2017) also when tested in produce (e.g. lettuce, jalapeño peppers), juices (apple, milk, etc.) and under simulated gastric conditions (Su and D'Souza 2013; Joshi *et al*. 2015; Falcó *et al*. 2019a).

These plant extracts were coupled to polysaccharides previously described to inactivate HAV, such carrageenans (Girond *et al*. 1991) and alginate, to produce edible films and coatings with improved technological (e.g. water vapour permeability, antioxidant activity) and antiviral activity, finally providing a better preservation of soft fruits (Fabra *et al*. 2018; Falcó *et al*. 2019b; Falcó *et al*. 2019c).

## HAV detection in food

Food represents a vehicle for HAV transmission to humans. In fact, HAV cannot replicate in host-free environments as water and food commonly resulting in very low titre as (cross-) contaminating particles. However, the HAV low infective dose poses a high risk to consumers irrespectively to the level of contamination. Given this panorama, sensitive and reproducible methods are needed to assess food safety even their development results challenging because of the wide range of food matrices and their characteristics that differently affect analytical procedures. Another critical aspect is the sampling procedure to be followed, especially during epidemiologic surveillance. As a general criteria the test sample has to be randomly selected and representative of the whole batch. Additional specific considerations for food sampling have been described elsewhere (i.e. analyse whole or chopped food items, consideration on viral internalization) (Bosch *et al*. 2011).

The detection of HAV in food samples has been achieved by molecular assays in which fit-to-purpose primers are designed to guarantee an absolute specificity. Even though conventional PCR remain useful as larger

PCR products can be obtained to confirm the specificity of the target, quantitative RT-PCR (RT-qPCR) assays have revolutionized viral detection in both clinical and environmental samples. The high sensitivity of RT-qPCR, the relative low cost of the analysis, and the enhanced specificity reached by the use of fluorescent probes, have made this technique the golden standard for HAV detection in food. Notably, RT-qPCR enables a quantitative viral detection of interest for hazard risk assessment analysis and its use in the improvement of public health measures and food-related regulations.

Therefore, several protocols have been reported and focused to different aims such as the applicability to various types of foods and the absence of potential inhibitors in molecular reactions. Unfortunately, a unique protocol encompassing all these ideal features is not available to date and the number of available protocols is overwhelming. As a breakdown, two recently released ISO procedures, ISO 15216-1:2017 and ISO 15216-2:2019, specify standardized protocols for quantitative and qualitative detection of HAV. These methods describe the concentration of viral particle from several high-risk food items, including soft fruits, leaf, stem and bulb vegetables, bivalve molluscan shellfish, bottled water and food surfaces, and the quantification of viral particles is achieved by RT-qPCR. Both ISO standards apply the same viral concentration and RNA extraction procedures. For viral detection, they both rely on one-step RT-qPCR using hydrolysis probes while differ on the use of a standard curve. Very recently, the ISO method 15216-1 has been validated in an international study involving 18 laboratories from 11 European countries. Moreover, this study provided useful analytical parameters such as the limit of detection (LoD), the limit of quantification (LoQ), repeatability and reproducibility (Lowther *et al.* 2019).

The extraction of HAV viral particles from food matrices can be defined as the separation and concentration of virus particles from the food matrix. Such methodologies usually consist of a virus elution step, following by a virus concentration and purification step designed to reduce sample volume and remove some of the food compounds while simultaneously recovering the contaminating viruses. Then, RNA is extracted by using an appropriate guanidine thiocyanate disruption method coupled to a silica adsorption membrane or, alternatively, by a magnetic beads-based method. According to ISO 15216, HAV in bivalve molluscan shellfish is extracted from the digestive glands using a treatment with a proteinase K solution. In a validation study using Pacific oysters, this procedure resulted in a LoD and a LoQ of 198 gc $g^{-1}$ (Lowther *et al.* 2019).

For HAV extraction from soft fruits, leaf, stem and bulb vegetables the ISO method recommends the elution by agitation followed by a precipitation with polyethylene glycol (PEG)/sodium chloride. Validated LoD and LoQ were 3·97 and 10·0 gc $g^{-1}$ in raspberries, 3·18 and 31·6 gc $g^{-1}$ in lettuce respectively (Lowther *et al.* 2019). For bottled water, adsorption/elution using positively charged membranes followed by concentration by ultrafiltration is recommended and validated LoD resulted in 0·40 gc $ml^{-1}$ and LoQ 1·00 gc $ml^{-1}$ (Lowther *et al.* 2019).

Losses of HAV can occur at several stages during viral concentration from sample and RNA extraction. To account for this, a process control virus is mandatory (ISO 15216–1 2017). As well, the incorporation of an RNA internal amplification control to monitor any potential matrix-derived inhibitory effect is highly recommended.

The Food and Drug Administration (FDA) includes in the Bacteriological Analytical Manual methods to detect HAV in shellfish and green onions (Goswami *et al.* 1993; Williams-Woods and Gary Hartman 2013). The viral concentration procedure from shellfish consists in the tissue homogenization using a sodium acetate buffer and water-saturated phenol solution, while for green onions a glycine buffer is coupled with ultracentrifugation. The RNA is isolated by phenol extraction from shellfish concentrates and by a column-based kit from green onions, and then quantified by RT-qPCR.

The Health Products and Food branch of the Government of Canada also provides standard methods for HAV detection and quantification in strawberries, green onions and bottled water included into the Health Canada's Compendium of Analytical Methods. For fresh green onions, viral particles are eluted by repeated pipetting/washing of the surface and concentrated by a PEG precipitation step. HAV RNA is extracted and purified using a combination of TRIzol®-chloroform and poly (dT) magnetic beads procedures. The Pathatrix™ equipment is instead used to force magnetic beads circulating throughout berry samples and viral particles captured onto a magnet. After subsequent washes with PBS buffer, HAV RNA is extracted with the QIAamp™ viral RNA mini kit.

HAV concentration from bottled water consists in filtering the sample through a positively charged membrane and eluting viral particles with a tryptose phosphate broth-glycine buffer. The eluates are further concentrated using Microsep 100™ or Centricon™ columns. The RNA is then extracted using the Qiagen RNeasy kit. For all procedures, the extracted RNA is finally subjected to a RT-qPCR for the final detection.

More recently, digital RT-PCR (RT-dPCR) has emerged as an alternative method for HAV detection in food.

RT-dPCR has shown comparable reproducibility, repeatability and sensitivity as RT-qPCR. The best advantages of the RT-dPCR in applied virology rely in its less proneness to PCR inhibitors, frequently found in RNA extracted from foodstuffs and environmental samples. This aspect is of extreme importance and made RT-dPCR a more robust method than RT-qPCR, when used without proper controls.

In RT-dPCR assay, the original RNA sample is partitioned on microfluidic chips or micro-droplets and each PCR amplification targets one or no copies of the nucleic acid. By using the same set of primers and probes as RT-qPCR, the absolute number of RNA copies is determined by the ratio of positive to total partitions by using binomial Poisson statistics (Dube *et al.* 2008). This approach allows the variation in amplification efficiency to be considered as negligible (Hindson *et al.* 2013) and the inhibition linked to matrix-type components, aspect of relevant importance in food and environmental virology, to be significantly reduced. Additionally, the absolute quantification of nucleic acids does not need a standard curve and may be a relevant alternative method to standardize quantification of HAV in foodstuffs. As a manner of fact, the RT-dPCR mitigated the impact of inhibitors, resulted in higher recoveries, and finally improved the detection of HAV in water, berries and lettuce samples (Coudray-Meunier *et al.* 2015; Fraisse *et al.* 2017; Sun, Bosch and Myrmel 2019). The very novel analytical methods applied to detect and characterize HAV in food and environmental samples rely on next generation sequencing (NGS) techniques. The wide potential of NGS includes the characterization of viral quasispecies heterogeneity (Yang *et al.* 2018) and the metagenomics approach represent a promising solution for detecting all viruses (virome), delivering genomic information and discovering unknown viruses (Nieuwenhuijse and Koopmans 2017; Yang *et al.* 2017). NGS have been successfully applied for amplification-independent detection of norovirus and HAV spiked in celery samples at low copy using both targeted and metagenomics approaches (Yang *et al.* 2017).

Notably, NGS was applied to detect HAV from frozen berries implicated in an outbreak. However, to overcome the anticipated low viral quantities, NGS was performed on amplicons obtained from HAV isolated and enriched by passaging in Frp/3 (Foetal rhesus monkey kidney, FRhK-4 derivative) cell culture (Chiapponi *et al.* 2014).

Beside these achievements, NGS presents significant limitations, especially when aimed to virome characterization. Contaminating genomes (e.g. human, bacteria) usually overshadow viral genomic material, thus sample preparation is a crucial step to enrich targeted nucleic acids. In addition, bioinformatics analysis and data interpretation hamper the use of NGS for routine and automated detection of HAV in food.

## Prevalence, levels and risk associated with HAV in foods

Despite HAV prevalence in food is relatively low, the risk associated with the infection is high and reported diseases and illness result relevant to public health.

However, considering that food can originate from very distant locations, used as ingredients in a wide variety of food items and contaminated in very low titre, outbreaks have been hardly linked to the contamination source timely.

The most common categories of food linked to outbreaks are shellfish, leafy greens, and fresh and frozen fruits, especially berries. However, any food can be implicated in outbreaks because of its potential cross-contamination.

Bivalve molluscan shellfish contamination is an important health and economic problem as the current commercial depuration procedures applied are not sufficient to eliminate HAV contamination (Polo *et al.* 2014a, 2014b). High levels of HAV in bivalve molluscan shellfish have been reported to date (Manso and Romalde 2013; Polo *et al.* 2015), with markedly varying prevalence among countries and in time (La Bella *et al.* 2017; Romalde *et al.* 2018) (Table 2). For instance, in systematic surveys performed in the last 20 years on bivalve molluscan shellfish from three estuaries in Galicia (Spain), the HAV prevalence sharply decreased throughout the years (from 40 to less than 8%) (Romalde *et al.* 2018).

HAV positive shellfish has been reported all over European countries, such as Greece (4% HAV prevalence), Spain (3–75%), United Kingdom (1%) (Formiga-Cruz *et al.* 2002; Pintó *et al.* 2009; Manso *et al.* 2010; Polo *et al.* 2015), Poland (7·5%) (Bigoraj *et al.* 2014) and Italy (0·9–23·2%) (Macaluso *et al.* 2006; Suffredini *et al.* 2014; Iaconelli *et al.* 2015; Purpari *et al.* 2019).

Globally, shellfish was tested positive for HAV as demonstrated by studies in the United States (4·4%) (DePaola *et al.* 2010), China (5%) (Ming *et al.* 2013), Thailand (3·8%) (Namsai *et al.* 2011), Japan (1·8%) (Hansman *et al.* 2008), Mexico (23·3%) (Parada-Fabian *et al.* 2016), Turkey (3·3%) (Yilmaz *et al.* 2018), Marocco (2·6%) (Benabbes *et al.* 2013), Tunisia (26%) (Elamri *et al.* 2006) and Vietnam (1·7%) (Suffredini *et al.* 2020).

Using data related to two shellfish-borne HAV outbreaks, the risk of infection after consumption of lightly cooked clams has been estimated as 0·05–0·54 (Pintó *et al.* 2009).

However, not only shellfish and bivalve mollusc have been detected positive for HAV, as many other food resulted responsible of outbreaks. The presence of HAV

**Table 2** Prevalence and levels of hepatitis A virus in bivalve molluscan shellfish

| HAV endemicity | Type of shellfish | Country | Number of samples | Point of sampling | Prevalence (%) | Levels | Samples associated with an outbreak | References |
|---|---|---|---|---|---|---|---|---|
| Intermediate | Coquina clams | Peru | 7 | Dispatch centre | 43 | $7{\cdot}5 \times 10^3$–$7{\cdot}3 \times 10^5$ gc $g^{-1}$ DT | Yes | Costafreda *et al*. (2006) |
| | Cockles, clams and oysters | Morocco | 77 | Production area | 2·6 | $10^2$ gc $g^{-1}$ DT | No | Benabbes *et al*. (2013) |
| | Oysters and coquina clams | Vietnam | 121 | Dispatch centre | 1·7 | $1{\cdot}3 \times 10^2$ gc $g^{-1}$ | No | Suffredini *et al*. (2020) |
| | Oysters, cockles and mussels | Thailand | 213 | Dispatch centres and production area | 3·7 | ND | No | Namsai *et al*. (2011) |
| | Mussels and clams | Tunisia | 23 | Production area | 26 | ND | No | Elamri *et al*. (2006) |
| | Mussels | Turkey | 736 | Production area | 3·3 | ND | No | Yilmaz *et al*. (2018) |
| Low | Oysters | Australia | 297 | Production area | No HAV detection | | | Torok *et al*. (2018) |
| | Mussels, oysters and clams | Italy | 336 | Production area (Class A and B)* | 0·9 | 5 to $7 \times 10^2$ gc $g^{-1}$ | No | Suffredini *et al*. (2014) |
| | Mussels and clams | Italy | 352 | Production area | 21·9 | <LoQ- $5 \times 10^3$ gc $g^{-1}$ DT | Samples collected after a contamination event | Suffredini *et al*. (2017) |
| | Mussels, oysters and clams | Italy | 253 | Production area (Class A and B)* | No HAV detection | – | | La Bella *et al*. (2017) |
| | Mussels and clams | Italy | 289 | Production area (Class A and B)* | 8·9 | <LoQ to $4{\cdot}2 \times 10^2$ gc $g^{-1}$ | | Fusco *et al*. (2019) |
| | Mussels, clams and cockles | Spain | 168 | Production area (Class B) | 10·1 | $9{\cdot}3 \times 10^3$ gc $g^{-1}$ DT | No | Polo *et al*. (2015) |
| | Mussels | Spain | 81 | Production area | 18·5 | $1{\cdot}1 \times 10^2$ to $4{\cdot}1 \times 10^6$ gc $g^{-1}$ DT | No | Manso and Romalde (2013) |
| | Mussels, oysters, clams and cockles | Portugal | 2000 | Production area (Class A, B and C) | 33 | ND | No | Mesquita *et al*. (2011) |
| | Oysters and mussels | France | 108 | Production area | 13 | ND | No | Le Guyader *et al*. (2000) |
| | Shellfish | Galicia (Spain) | ND | Production area | 8–40 | – | Partially | Romalde *et al*., (2018) |

Gc, genome copies; DT, digestive tissue; ND, not determined.

*EU regulations classified shellfish harvesting areas into A, B or C category on the basis of *Escherichia coli* levels as follows: A (≤230 CFU *E. coli*/100 g shellfish), B (230–4600 CFU *E. coli*/100 g shellfish), C (4600–46 000 CFU *E. coli*/100 g shellfish).

has been confirmed in several food matrices worldwide even limited data are available as quantitative levels. For instance, in Mexico HAV was present in 28·6, 8·3 and 36·4% of spinach, parsley and purslane respectively. Prevalence of 28·2% was previously reported with titre from $2{\cdot}8 \times 10^2$ to $2{\cdot}4 \times 10^3$ gc $g^{-1}$ (Felix-Valenzuela *et al.* 2012; Parada-Fabian *et al.* 2016). A recent study performed on fresh produce in Egypt found that HAV was present in 25·8% of samples at around $6{\cdot}4 \times 10^3$ gc $g^{-1}$ (Shaheen, Elmahdy and Chawla-Sarkar 2019). Similarly, various frozen berries, frozen diced potatoes and frozen diced apple were tested as positive in China (2·8% of total) (Fang *et al.* 2016), as well as strawberries (0·001%) in South Korea (Shin *et al.* 2019). Of note, a recent study reported that the 18% of raw and processed foods of animal origin traded in Brazilian borders were tested positive for HAV (Pereira *et al.* 2018). In Australia, the prevalence of HAV in berries and leafy greens at retail was estimated to be <2%, with no virus detected in produce during the survey. The risk associated with fresh Australian berries and leafy greens, evaluated using standard codex procedures and the Risk Ranger tool, was deemed to be low (Torok *et al.* 2019).

In Europe, HAV has been detected in ready-to-eat salads in Italy (1·9%) (Terio *et al.* 2017), while berry samples ($n = 120$) collected at point-of-sale were negative for HAV (Maunula *et al.* 2013). A recent study performed on berries by different service laboratories from 2009 to 2016 detected HAV RNA in 2 of 2015 samples (0·01%; Li *et al.* 2018).

Whichever is the food item pointed out as the source of HAV contamination, either related or not to an outbreak or investigated by molecular methods (thus, not necessarily representing infectious particles), the presence of HAV RNA typically suggests that faecal contamination has occurred somewhere along the food chain from farm to fork. In this scenario and given the low infectious dose of HAV, the adoption of a presence/absence criterion has been proposed as the most appropriated to guarantee food safety (Suffredini *et al.* 2014).

## Assessing HAV infectivity in food

The assessment of infectivity is a major requirement in several areas of virology, including clinical and environmental diagnosis, ecology, risk assessment and food control. Since HAV was first adapted to cell culture by Provost and Hilleman (1979) and an efficient plaque assay developed using an isolate (HM-175) lytic for FRhK-4 cells (Cromeans *et al.* 1987), many efforts have been made to expand the knowledge on methods to assess infectivity. In fact, the persistent replication as well as the cytopathic effect of the isolated strain is not a common feature of other HAV strains, especially the wild types (wt). A cell line (Huh7-A-I) permissive for wt HAV infection has been described and successfully tested for replication of a natural isolate from human stool (Konduru and Kaplan 2006). Viral replication of wt isolates from contaminated molluscs has been reported by using an integrated cell culture PCR (ICC RT-qPCR) method using FRhK-4-derived Frp3 cells. This ICC RT-qPCR assay was applied to detect infectious HAV in naturally contaminated molluscs that may not cause cytopathic effect in cell culture (De Medici *et al.* 2001; Chironna *et al.* 2002; Croci *et al.* 2005).

On the contrary, fast and sensitive analytical molecular procedures are useful to identify HAV in food and environment samples, but do not inform on their infectivity. In fact, the viral genomes detected by molecular techniques do not represent the infectivity of a given sample, being the nucleic acids of inactivated and defective viral particles quantified along with infective virus. Moreover, the persistence of HAV RNA in the environment may be much greater than that of the corresponding infectious viruses (Hewitt and Greening 2004; Butot, *et al.* 2008; Butot *et al.* 2009; Randazzo *et al.* 2018a, 2018b). Thus, it results of extreme interest to develop methods able to assess viral infectivity in food, water and environmental samples with the final goal of improving the risk assessment in terms of public health.

To date, a reliable method to assess HAV infectivity in food matrixes remains a challenge and some techniques based on the integrity of the capsid are in use as indirect approximations. These approaches assume that the detection of RNA from intact viral particles could be used to estimate infectivity since viruses need an intact capsid to be infective.

The enzymatic digestion with RNase prior to nucleic acid extraction is a simple approach to remove amplification signals of exposed genomes and it has been successfully applied to detect potentially infectious HAV particles in fresh produce (Marti *et al.* 2017).

Similarly, alternative approaches based on viability markers applied as pretreatments before the RT-qPCR have been optimized to detect infectious HAV particles in food matrices. Firstly implemented for bacterial pathogens by Nogva *et al.* (2003), viability PCR (vPCR) implies the use of viability markers able to unspecifically intercalate exposed nucleic acids and, thus prevents PCR amplification of microorganisms with damaged or altered membrane or capsids.

Viability markers can be grouped into photoactivatable dyes (i.e. propidium monoazide, PMA, and ethidium monoazide, EMA) and metal compounds (i.e. platinum and palladium compounds). The former need a photoactivation step to covalently bind viral RNA, resulting more

expensive and time-consuming than metal compounds. However, to boost the final efficacy of a viability marker, optimization tests should be carried out assessing variables such as the amplification target, the matrix, the addition of surfactants and the inactivation procedure.

**Table 3** Application of intercalating dyes for the detection of potentially infectious hepatitis A virus under different inactivation treatments

| Treatment | Intercalating dye pretreatment | Matrix | References |
|---|---|---|---|
| Heat | | | |
| 60, 72 and 95°C (15 min) | PMAxx (50 $\mu$mol $l^{-1}$)// Triton X-100 (0·5%) | Cell culture suspension | Randazzo *et al.* (2018b) |
| 60, 72 and 95°C (15 min) | PMAxx (100 $\mu$mol $l^{-1}$)/ Triton X-100 (0·5%) | Berries | Chen *et al.* (2020) |
| 68, 72 and 80°C (1, 5, 10 and 20 min) | EMA (20 $\mu$mol $l^{-1}$)/ IGEPAL CA-630 (0·5%) | Cell culture suspension | Coudray-Meunier *et al.* (2013) |
| 70, 85 and 99°C (5 min) | PMA (200 $\mu$mol $l^{-1}$)/ Triton X-100 (0·5%) | Cell culture suspension | Fuster *et al.* (2016) |
| 99°C (5 min) | PMA (50 $\mu$mol $l^{-1}$) | Cell culture suspension | Sánchez *et al.* (2012) |
| 99°C (5 min) | PMA (50 $\mu$mol $l^{-1}$)/ Triton X-100 (0·5%) | Lettuce wash water, vegetables and shellfish | Moreno *et al.* (2015) |
| 99°C (5 min) | PMAxx (50 $\mu$mol $l^{-1}$)/ Triton X-100 (0·5%) | Vegetables and shellfish | Randazzo *et al.* (2018b) |
| 95°C (10 min) | Reagent D | Cell culture suspension | Monteiro and Santos (2018) |
| Chlorine | | | |
| 0, 2·5, 5 and 10 mg $l^{-1}$ (30 min, RT) | PMA | Cell culture suspension | Fuster *et al.* (2016) |
| High pressure processing | | | |
| 500 MPa (15 min) | PMA | Cell culture suspension | Sánchez *et al.* (2012) |
| 500 MPa (15 min, 29°C) | PMAxx | Cell culture suspension | Randazzo *et al.* (2018c) |

Viability RT-qPCR assays have been used to estimate infectious HAV exposed to thermal treatments. The have been optimized in viral suspensions, tested in inoculated food concentrates (including lettuce, spinach, parsley, cockles, coquina clams, mussels, oysters and berries) and successfully applied in naturally contaminated river, sewage and reclaimed waters (Moreno *et al.* 2015; Fuster *et al.* 2016; Randazzo *et al.* 2018b, 2018c; Chen *et al.* 2020) (Table 3).

Recently, PMAxx photoactivatable dye (Biotinum, CA) better discriminated thermal and high pressure treated HAV compared with a metal compound (platinium(IV) chloride, $PtCl_4$). Intriguingly, opposite results were obtained for hepatitis E virus, confirming the relevance of the test parameters for the outcomes of the viability molecular assay, specifically the viability marker (Randazzo *et al.* 2018b).

On the other hand, some downsides of this approach have been noticed, particularly the lack of the complete suppression of RT-qPCR signal from inactivated viruses. This has been observed in food matrices (e.g. shellfish) and environmental waters, as well as when viral inactivation is achieved by chemical or natural compounds (e.g. chlorine, catechin) or high pressure treatments (Fuster *et al.* 2016; Falcó *et al.* 2017; Randazzo *et al.* 2018a, 2018b).

## Conclusions and future perspectives

Despite the consistent improvements of hygienic conditions worldwide and the availability of HAV vaccines, there is still a notable burden of hepatitis A disease associated with food consumption. While the development of a robust cell culture method could represent a tremendous step forward for the assessment of HAV infectivity in food, viability RT-qPCR is a rapid and cost-effective assay that provides a more accurate measure of the risk associated with contaminated food and waters than RT-qPCR alone.

Next generation sequencing techniques are promising tools to detect and characterize HAV in food and environmental samples, even further developments in virus-specific nucleic acid extraction methods, bioinformatics analysis and data interpretation are needed before their application for food safety and surveillance purposes.

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